Structural basis of a Temporin 1b analogue antimicrobial activity against Gram negative bacteria determined by CD and NMR techniques in cellular environment

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Abstract

We here report an original approach to elucidate mechanisms of action of antimicrobial peptides and derive crucial structural requirements for the design of novel therapeutic agents. The high resolution structure of TB_KKG6A, an antimicrobial peptide designed to amplify the spectrum of action of Temporin B, bound to *E. coli* is here determined by means of CD and NMR methodologies. We have also defined, through STD analysis, the residues in closer proximity to the bacterial membrane.

AMPs are produced by all organisms, eukaryotes and prokaryotes, either ribosomally or by multifunctional peptide synthetases[1].
The detailed mechanism by which AMPs work is far from being understood, although several hypotheses have been proposed[2]. AMPs can either target bacterial membranes or interact with the bacterial cytoplasmic content, causing the loss of vital function for bacteria and ultimately bacterial death. Partition constants calculated for AMPs suggest the higher affinity of peptides for bacterial vs mammalian membranes and also indicate that a very high local concentration of peptides on bacterial membranes is compatible with antibacterial activity[3]. The structures of AMPs have been so far derived only in presence of bacterial membrane model systems, such as SDS and LPS. It has been demonstrated that the three-dimensional structure of peptides, obtained by solid state NMR in the presence of lipids mixtures, depends on the composition of the mixture[4]. Gain of details of the peptide interactions with intact cellular membranes, i.e. with whole bacterial cells, during the first stages of their activity represents a key step to fully understand their mechanism of action and derive crucial structural requirements for the design of novel therapeutic agents. Recent studies carried out by time-lapse fluorescence imaging studies on a melittin analogue with *E. coli* cells and synthetic membranes revealed a different behaviour of the peptide in the two different experimental systems[5]. Binding of the PMAP-23 peptide to *E. coli* cells has been investigated by fluorescence[6]. Structural studies of the AMPs magainin 2 and cecropin A by CD in the presence of *E. coli* cells and LPS have only recently been reported[7]; the results obtained in the two different experimental systems are comparable but not superimposable, likely because of the different chemical composition of the two systems. Such studies allowed the determination of the peptide secondary structure in presence of cells but did not yield details at molecular level on the peptide three-dimensional arrangement.
Recently we reported the design and characterization of TB_KKG6A[†8], a new analogue of TB. TB is a member of the temporin family, ribosomally synthesized peptides, initially isolated from the skin of *Rana temporaria* that represents the largest family of AMPs in amphibian (over 50 members)[9]. They are peptides composed of 8–14 amino acids amidated at the C-terminus and present a low net positive charge (from 0 to +3) at neutral pH. They are mainly active against Gram+ bacteria; few members of the temporin family, such as TL and T1DRa, efficiently kill Gram- bacteria but also show haemolytic activity[10].
Temporins, as most of the AMPs, do not show conformational preferences in aqueous buffers but, being highly membrane-active, have been hypothesized to fold upon interaction with bacterial membranes[8, 11, 12, 13, 14]. TA and TL assume helical conformation in SDS, DPC and LPS micelles[12, 15, 16]. Interestingly, the structure of TA and TL in SDS and DPC is monomeric, while in LPS both oligomerize in a head to tail fashion. NMR data on TB show that it does not fold in presence of LPS, likely because of self association. Combination of TB with sub-inhibitory concentrations of TL seems to prevent TB oligomerization on LPS, although the peptides are not at NMR measurable distances[14, 17].
The peptide TB_KKG6A contains one point mutation (G6A) within the TB sequence and two extra lysines at the N-terminus; it is active either against Gram+ and Gram- bacteria with a total lack of haemolytic activity. It is also able to down-regulate the expression of pro-inflammatory chemokines in IB3-1 cells infected with *P. aeruginosa*[18]. We showed via CD and NMR that, upon binding to

LPS micelles, the peptide assumes a monomeric helical structure composed of two helical segments. The two extra Lys residues lies on the external surface of the LPS, anchoring the peptide to the bacterial membranes while Lys10 has a key role in penetrating the micelle[8].

Here, to gain structural details at the base of TB_KKG6A activity, we report the structural characterization via CD and NMR of TB_KKG6A bound to the Gram- bacterium *E. coli*. Experiments were carried out suspending cells respectively in phosphate buffer and in PBS as these conditions are compatible with the experimental measurements, and with *E. coli* survival[7] (Supplementary Figure S1The cell viability was also evaluated in the presence of TB_KKG6A in PBS (Supplementary Figure S1). The number of cells remained steady in the two first hours, but after this time cells lost their ability to duplicate when resuspended in LB

The interaction of TB_KKG6A and of TB with *E. coli* was initially investigated by CD (Figure 1) as recently described[7].

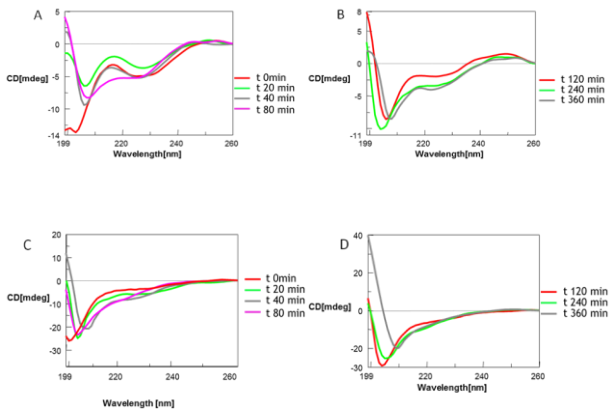

Figure 1. CD spectra recorded at a 10μM concentration of TB_KKG6A (Panels A and B) and TB (Panels C and D) incubated with *E. coli* cells (OD600 0.1) in phosphate buffer 10mM, pH7.0.Spectra shown are the results of the subtraction of the cell spectra to the cell+peptide mixture spectra.

Time course experiments carried out in a 6 hours time span revealed that TB_KKG6A, which appears random coil and is likely in an oligomeric state in phosphate buffer (as suggested by NMR, see below), upon interaction with cells folds into a helix. The CD spectra reach the maximum intensity within 80 minutes of incubation and after this time, the intensity of the band around 224 nm tends to decrease.

Time course experiments revealed that the peptide does not show conformational preferences when incubated with the cytoplasmic cell content obtained after sonication and centrifugation of bacteria, (Supplementary Figure S2) suggesting that the folding processes are induced by the bacterial outer membrane components.

By contrast, TB, which in solution appears in a random conformation, in *E. coli* suspension remains mostly disordered (as observed also in the presence of the cells cytoplasmic content), although a little change in the position of minima appears after 40 minutes of incubation with cells (Figure 1). Thus, TB either shows a very low affinity for bacteria or aggregates on *E. coli* cells or is trapped by the cell membrane, as hypothesized for another temporins member, TA, which is also inactive against Gram- bacteria.

Sequence specific assignments of TB_KKG6A ^{1}H chemical shifts (Table S1) and TB in the presence of *E. coli* were achieved analyzing ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H NOESY spectra[19] exploiting the previous reported assignments obtained for the monomeric peptide in presence of LPS[8,15]. Such chemical shifts do not change significantly in the presence of cells. Chemical shifts of both peptides assume typical random coil values along the entire sequence; nonetheless, while 2D tr-NOESY spectra of TB appear poor of cross-peaks, those of TB_KKG6A show a number of significant interresidues NOEs, indicating a cell-induced structural transition from random coil to a folded conformation.

Accordingly, D_{trans} measured on the peptide in presence of cells has a value of 1.49 ± 0.03 x 10^{-10} m^2/s. The diffusion coefficient measured in the cell suspension is as expected slower than the one measured in simple water (ref 8) but faster than the one measured in PBS and ascribed to an aggregated form of the peptide. In fact, in buffer solution, the NMR spectra clearly indicate that TB_KKG6A is mostly aggregated (see SI); accordingly, in such conditions D_{trans} (see Ref.6 in SI) yields a value of 0.83 ± 0.01 x 10^{-10} m^2/s. Aggregation of the peptide in PBS was confirmed by DLS measurements, carried out for the peptide TBKK_G6A (Supplementary …)

Overall, the DOSY and the chemical shift data are reasonably in agreement with a monomeric form of the peptide in a much more viscous environment.

In presence of *E. coli*, TB_KKG6A residues of the Pro3–Ser11 segment are characterized by strong short range HN_i-HN_{i+1} and medium range NOEs, in particular HN_i-HN_{i+2}, $H\alpha_i$–HN_{i+3}, and $H\alpha_i$–$H\beta_{i+3}$ correlations, typical of a helical conformation[19].

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A total of 189 NOEs was obtained from tr-NOESY spectra of TB_KKG6A; these NOEs resulted in 143 meaningful distance (113 inter-residues, 30 intra-residues) and 85 angle constraints which were used to generate a total of 100 structures, among which the 20 with the lowest target function were selected and energy minimized. Noteworthy, all the recorded NOEs have been used to determine the monomeric structure of the peptide and no intermolecular NOE have been detected. The absence of long range NOEs connecting residues which are far apart in the structure suggests that the peptide when bound to *E. coli* membrane very likely does not undergo self-association .

The obtained structures satisfied the NMR constraints, with no violations greater than 0.2 Å (Table S2). The NMR structure of TB_KKG6A bound to *E. coli* is well defined as the RMSD for residues Pro3-Ser11 is 0.29Å and shows that the peptide folds in a regular helix, including two turns from Pro3 to Leu12 (Figure 2).

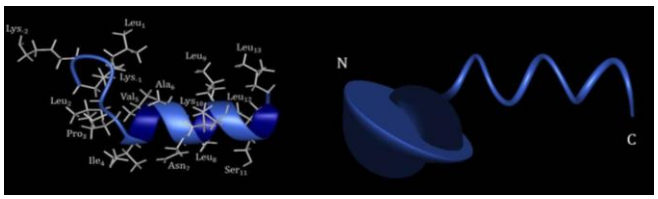

Figure 2. Left. The side-chains orientation of a representative NMR structure of TB_KKG6A in presence of *E. coli* cells. **Right.** Sausage representation of NMR ensemble of structures of TB_KKG6A in presence of *E. coli* cells.

One side of the helix (Figure 3),contains Pro3, Ala6 together with the two polar aminoacids (Asn7 and Ser12) and the positively charged Lys10 with its aliphatic chain. The other side of the helix is exclusively hydrophobic and constituted by Val5, Leu8, Leu9 and Leu12. Lys_{-2} and Lys_{-1} at the N-terminus, appear mostly structurally disordered, while Lys10 is more rigid and located on the second turn of the helix. This indicates that the relative positions of Lys10 and the two N-terminal lysines are not fixed, possibly because Lys_{-1} and Lys_{-2} form electrostatic interactions with phosphates of LPS chain protruding from the outer cellular leaflet. Concurrently, Lys10 is part of the helix surface, formed also by Pro3, Ala6 and Asn7 (Figure 4), directly interacting with the bacterial outer membrane.

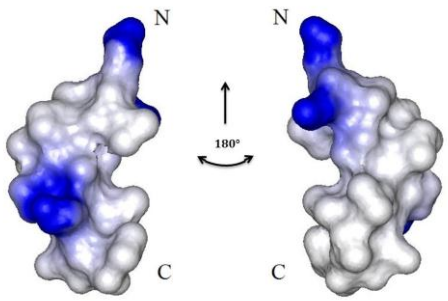

Figure 3. Representation of the electrostatic surface potentials of TB_KKG6A bound to *E. coli* cells. The hydrophobic surfaces are colored in light gray, positively charged residues in blue.

Modes of TB_KKG6A interaction with *E. coli* cells are confirmed by the STD[20] in which (Figure 4A) unambiguous STD effects could be assigned to Pro3, Ala6, Asn7 and Lys10.
In particular, Pro3 Hδs, Ala6 Hα and Hβs, Asn7 Hβs and Lys10 Hγ showed similar STD effects of about 50% (Figure 4B), suggesting their closer proximity to bacterial cell membranes. Differently, Lys_{-2} and Lys_{-1} do not show STD effects.

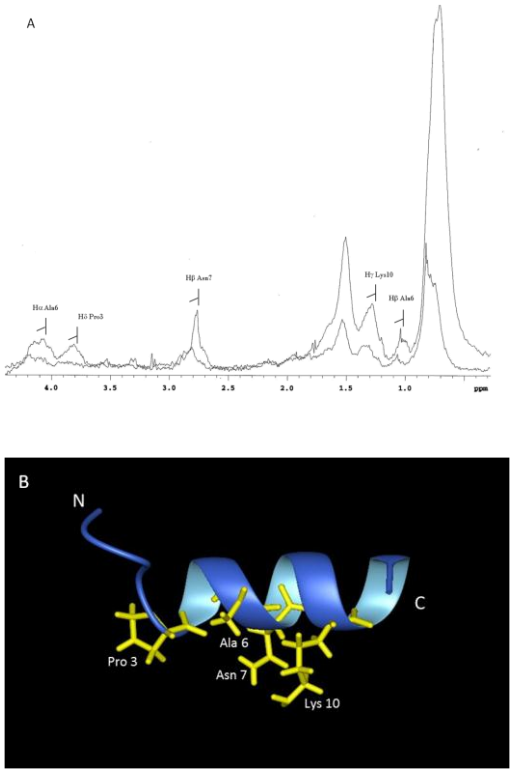

Figure 4. A: Superposition of the STD ^{1}H-NMR spectrum and the reference ^{1}H-NMR spectrum of TB_KKG6A in *E. coli* showing STD effects for side-chain protons. Unambiguous side-chain signals are assigned. **B:** Mapping onto a TB_KKG6A representative conformer of the residues that interact with the *E. coli* cell membrane.

These data together with the CD results suggest a mechanism in which the disaggregation and folding processes of TB_KKG6A are induced by the bacterial outer membrane components. As other AMPs including polymixin B, pardaxin and MSI-594[12,23] TBKKG6A establishes with LPS strong interactions in a monomeric form. In other cases, as for TL, dimerization occurs to widen the peptide/LPS interaction surface. Peptides unable to kill Gram-bacteria either show very low affinity toward the LPS, as TB, or are trapped into it, as TA[15,16].The peptide TB_KKG6A through Lys_{-1} and Lys_{-2} amino groups anchors the negatively charged phospholipids and specifically binds to the bacterial membranes through Pro3, Ala6, Asn7 and Lys10 side chains. Pro3, and Ala6 likely establish hydrophobic interactions with the bacterial membrane, analogously to what observed in presence of LPS micelles [8]. Lys10 side chain may either interact with the lipids of the membrane through its long aliphatic chain, being inserted into the membrane, and/or may interact through the amino group with negatively charged phosphates. The presence of Lys10 on the same side of the helix of Pro3, Ala6, together with STD data suggesting a strong interaction between the side chain of Lys10 and the bacterial cells, is compatible with the formation of a disordered toroidal pore. Such structures have been hypothesized for melittin and magainin H2: in these peptides the charged residues were observed to point either into the pore or towards the water layer[21,22].
Accordingly, our recent characterization of TB_KKG6A in presence of LPS micelles[8] also suggested that the augmented activity of this

peptide could find an explanation in its ability to form strong interactions with LPS thanks to the increased positive charge at the N-terminus.

Figure 5 reports the superposition of the two structures obtained in the two different systems, i.e. cellular context and LPS. While both systems induce the transition from a random coil to a helical structure, the two structure appear clearly different. In particular, in the presence of intact *E. coli* TB_KKG6A shows an uninterrupted helical segment while in presence of *E. coli* LPS folds into a kinked helix: a first helical segment encompasses residues Pro3 to Asn7, followed by a kink including residue Leu8 and by a second helical region from residue Leu9 to Ser11.. As it is shown clearly in Figure 5, Pro3, Ala6 and Asn 7 side chains, involved in the interaction with LPS and the intact E,Coli membrane, assume very similar orientations in the two environments. Differently, Lys10 side chain, which in LPS adopts two main different orientations, in cellular environment assume a single preferred conformations, significantly different from those observed in LPS. These structural features support the hypothesis that Lys10 may play a major role in the membrame permeation and indicate that the data obtained in LPS, that represent a valuable model tool to mimic the bacterial membrane, can be profitably complemented by structural data obtained in the presence of cells.

The data here presented supply extra detailed structural information about peptide-cellular membrane interactions. The results confirm the identity of the residues responsible for the interactions with outer leaflet and underline how the presence of concentrated positive charges is pivotal to the activity against Gram- bacteria. These results are further supported by literature data, showing that natural and also synthetic peptides with exposed positive charges, as protegrins and most of peptides/proteins possessing a gamma core motif[23], are highly active against Gram- bacteria.

Conclusions

In the present study, we have determined, by means of CD and NMR methodologies applied in cellular environments, the high resolution structure of an antimicrobial peptide, designed to amplify the spectrum of action of TB, bound to Gram- *E. coli* membrane. We have also defined, through STD analysis, the peptide surface in closer proximity to the outer membrane. These data provide the high resolution molecular details of an AMP directly bound to the bacterial cells, complementing our recent structural characterization of TB_KKG6A in the presence of LPS membrane model system. In particular, the presence of a positively charged patch seems to be required to increase the affinity of AMPs toward Gram- bacterial cells; furthermore the lack of a marked amphipaticity helps to prevent the aggregation of the peptide on cell membrane.

Notes

†**TB_KKG6A sequence** = $K_2K_{-1}LLPIVA_6NLLKSLL$

Abbrevations: AMP = Antimicrobial peptide; TA = Temporin-1a; TB = Temporin-1b; TL = Temporin L; T1DRa = Temporin 1DRa; $Gram^+$ = Gram positive; $Gram^-$ = Gram negative; DPC = Dodecylphosphocholine; SDS = Sodium dodecyl sulfate; LPS = Lipopolysaccharide,is the main component of the Gram- bacteria outer leaflet and therefore it is considered a reliable model system for bacterial membranes; CD = Circular dichroism; NMR = Nuclear magnetic resonance; STD = Saturation Transfer difference; NOE = Nuclear Overhauser Effect; D_{trans} = Translational Diffusion Coefficient; RMSD = Root Mean Square Deviation.

Methods

For methods see Supporting Information.

Associated Content

Supporting Information

Supplementary figures and table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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